ZOOLOGICAL SCIENCE 11: 77–82 (1994)


# Regional Differences in Granulosa Cells of Preovulatory Medaka Follicles

Takashi Iwamatsu[1]*, Seiko Nakashima[1], Kazuo Onitake[2], Arito Matsuhisa[1] and Yoshitaka Nagahama[3]

[1]*Department of Biology, Aichi University of Education, Kariya 448,* [2]*Department of Biology, Faculty of Science, Yamagata University, Yamagata 990, and Reproductive Laboratory, National Institute for Basic Biology, Okazaki 444, Japan*

**ABSTRACT**—The regional differences in morphology and steroid production of granulosa cells of the animal and vegetal hemispheres of the preovulatory medaka (*Oryzias latipes*) follicles were investigated. Granulosa cells in the animal pole region were distinguishable from those in the vegetal pole region in their shape and distribution. The distribution of tall granulosa cells was more compact in the vegetal pole area than in the remaining areas. The gonadotropin-induced production of 17$\alpha$,20$\beta$-dihydroxy-4-pregnen-3-one by granulosa cells alone was at a level sufficient to induce *in vitro* maturation of the oocyte. Production was significantly greater in the vegetal hemisphere than in the animal hemisphere. These results indicate that medaka follicles have morphological and physiological differences in granulosa cells corresponding to regional differences along the animal-vegetal axis of the oocyte.

## INTRODUCTION

Eggs of oviparous fishes generally have distinct polarity. The polarity reflects the functional organization of the cellular constituents necessary for development. It must be determined by the interaction of the oocyte with follicle cells, especially the granulosa cells that are in contact with the oocyte during oogenesis. Accessory structures such as attaching filaments, which are located at the animal or the vegetal pole side of the egg membrane (chorion) have been found in a great number of teleost fishes (cf. [14]). In medaka oocytes, the difference in the distribution of accessory filaments on the chorion is detectable in the early stages of oogenesis [5]. The differences in their morphology and distribution seem to be established by the interaction with granulosa cells.

In some teleosts, one of the maturation-inducing steroids (MIS: [11, 16]), 17$\alpha$,20$\beta$-dihydroxy-4-pregnen-3-one (17$\alpha$, 20$\beta$-diOHp: cf. [13]) is produced and secreted by follicle cells in the presence of gonadotropin. The maturation of medaka oocytes *in vitro* is also induced by the same steroid, which is produced by granulosa cells [6, 8–10, 15]. It is generally believed that whole surface of the oocyte is simultaneously evenly stimulated by MIS from the granulosa cells. However, there is no evidence that this is true.

In the medaka MIS is produced by granulosa cells with probably little or no participation of the thecal cells. Therefore, the spatial and functional differences in granulosa cells may be very important for the establishment of oocyte polarity and oocyte maturation in this fish. For this season, we are interested in the regional differences in steroidogenesis, particularly MIS production, and cytological characteristics of granulosa cells surrounding the oocyte before maturation. At present there is little information on the differences in the distribution between the animal and vegetal hemispheres and the structure of granulosa cells surrounding fully-grown oocytes.

We investigated the local differences in the structure and function of granulosa cells of the medaka follicle. The results indicated that granulosa cells had regional differences in morphology and steroid production along the animal-vegetal axis of the fully-grown oocyte.

## MATERIALS AND METHODS

*Follicle preparation*

Preovulatory medaka follicles were isolated from the ovaries of mature females which had spawned every day under the light- and temperature-conditions controlled to induce reproduction. The follicles were removed from the ovaries by using fine forceps under a binocular dissecting microscope (×20). The follicles were handled in saline [3]. Fine watchmaker's forceps were then used to peel the thecal cell layer and the basement membrane off from follicles starting at the vegetal pole area (VPA) identified by the long attaching filaments [4]. These oocytes surrounded by a granulosa cell layer alone were cut precisely with small scissors into two halves, the animal and the vegetal hemispheres. The ooplasm was removed from the hemispherical chorion within the granulosa cell layer by washing the specimens in saline with a small pipette. Thus, preparations consisted of granulosa cell layers on the chorion hemispheres.

*Follicle incubation*

Ten of the animal or vegetal hemispheres prepared above were placed separately in 1 ml of culture medium (Earle's Medium 199, Dainippon-seiyaku, Osaka) in each well of a 24-well culture dish (Cell Wells: Corning, N.Y.) and incubated for 24 hr at 27°C.

*Observations by fluorescence and electron microscopy*

Immediately after removal of the thecal cell layer and the basement membrane, oocytes surrounded by a granulosa cell layer

Accepted December 14, 1993
Received October 20, 1993
* To whom reprint requests should be addressed.

alone were pre-fixed in 4% glutaraldehyde, rinsed in 0.1 M phosphate buffer (pH 7.4) and post-fixed in osmium tetroxide at 4°C for 2 hr. These samples were washed in 0.01 M phosphate buffer (pH 7.4), dehydrated in a graded series of acetone, dried according to the critical point method with $CO_2$ and coated with gold. They were observed with a JEOL scanning electron microscope (SEM).

For transmission electron microscopy (TEM), the similarly fixed samples were dehydrated in ethanol followed by propylene oxide and embedded in Epon 812. Ultrathin-sections were observed after staining with lead citrate and uranyl acetate.

*Measurement of steroids*

Animal and vegetal hemispheres prepared above were incubated in the presence of 100 IU pregnant mare serum gonadotropin (PMSG) or 100 ng/ml progesterone for 24 hr at 27°C. One milliliter of culture medium from each well was then collected and assayed to compare the production of 17$\alpha$,20$\beta$-diOHp and estradiol-17$\beta$ ($E_2$) by granulosa cells of the two hemispheres. 17$\alpha$,20$\beta$-DiOHp and $E_2$ were measured in the samples of culture medium using radioimmunoassay (RIA) as described in a previous report [9, 10]. The sensitivity of each assay was 30 pg/ml.

All experiments were performed in triplicate and the data were analysed by statistical comparison (the Student's *t*-test) with control values.

## RESULTS

*1 Morphology of granulosa cells*

As shown in the TEM images (Figs. 1 and 2), granulosa cells on the chorion of preovulatory oocytes differed in shape and number between the animal and vegetal hemispheres. Short cylindrical granulosa cells (diameter ca. 8 $\mu$m, height 13 $\mu$m) were aligned in a monolayer on the chorion (Fig. 1), except in the vegetal pole area(Fig. 2) (VPA, diameter ca. 430 $\mu$m, [5]). The nucleus was located centrally in these short cylindrical cells. The cells contained well developed

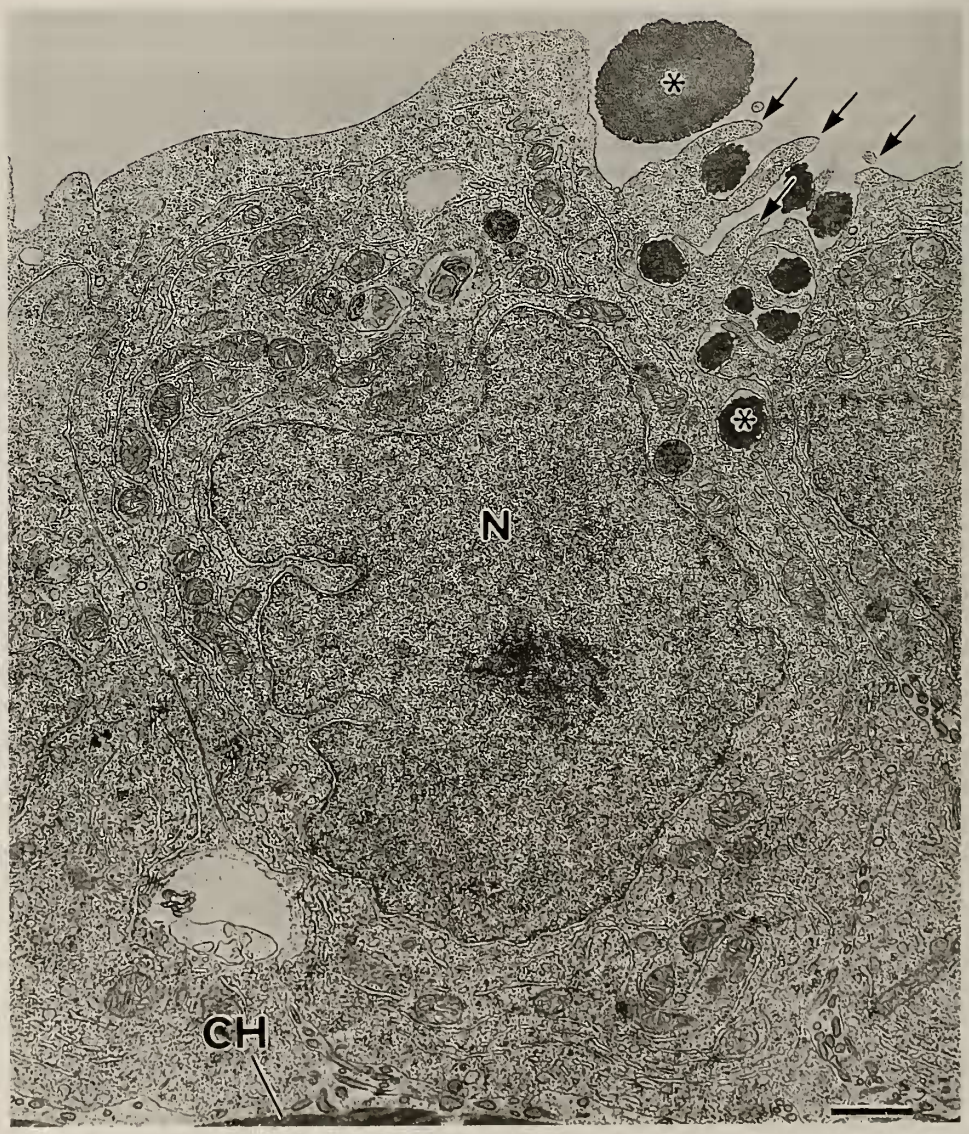


FIG. 1. Granulosa cells in the animal hemisphere of the preovulatory medaka follicle. The granulosa cell contains many mitochondria, rough endoplasmic reticula and Golgi apparatus in the cytoplasm. Note microvilli (arrows) without these organelles at the apical surface of the cell. Asterisks, non-attaching filaments; CH, chorion; N, nucleus. (bar 1 $\mu$m)

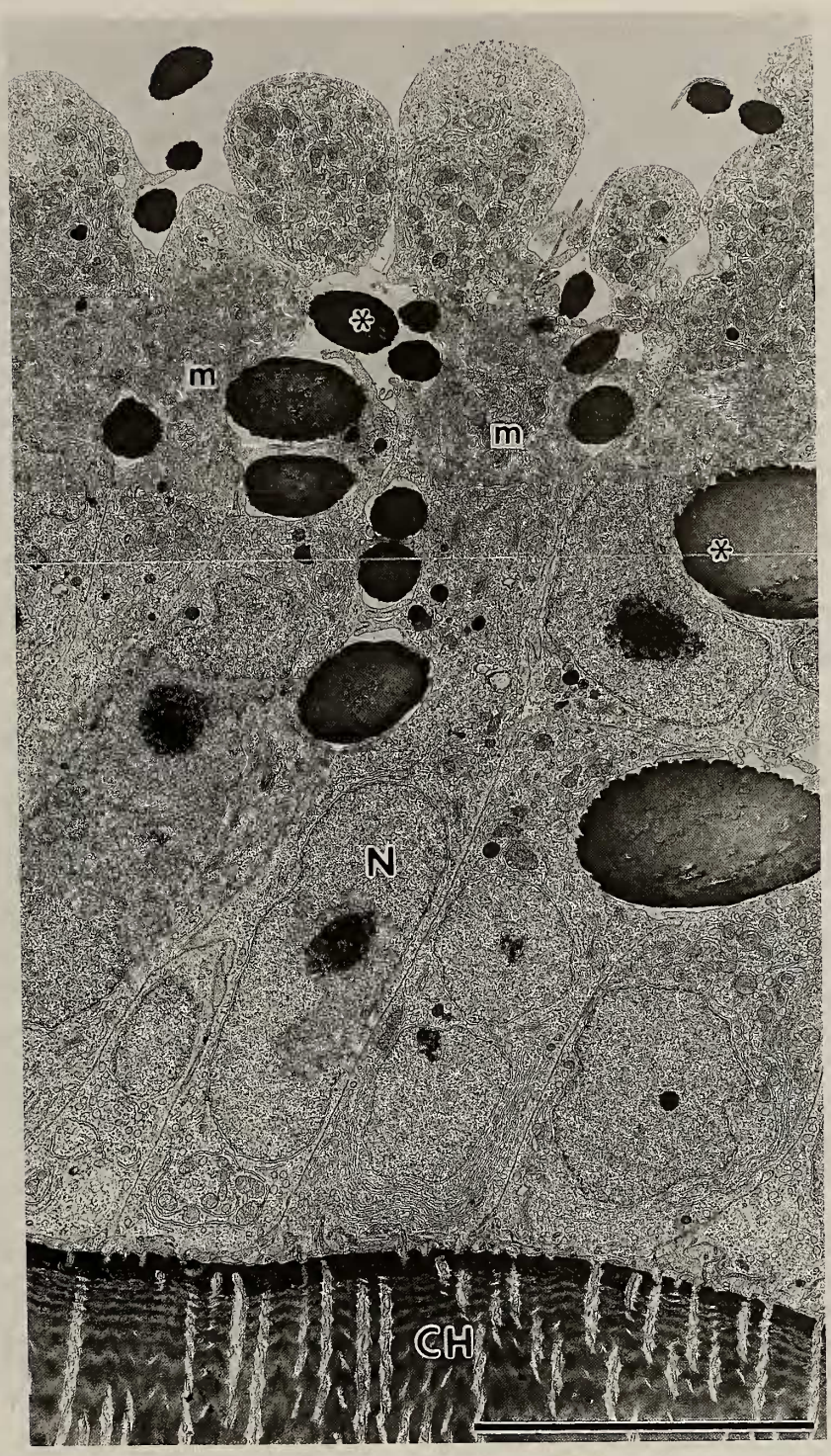


FIG. 2. Granulosa cells in the vegetal pole area of the preovulatory medaka follicle. Tall granulosa cells contain a mitochondrial mass (m), dilated endoplasmic reticula (vesicles), and developed Golgi lammellae in the apical region of the cell. Note the breb-like apices of the granulosa cells which contain many cytoplasmic inclusions. Asterisks, attaching filaments; CH, chorion; N, nucleus. (bar 10 $\mu$m)

rough endoplasmic reticula (ER), some of which were dilated, mitochondria with an electrondense matrix, Golgi complexes with very small vesicles, and large lysosomal vesicles. The granulosa cells in the VPA were slimmer and taller (diameter ca. 5 $\mu$m, height ca. 37 $\mu$m) than those in the animal hemisphere. The nucleus was basally located near the chorion, and the conspicuously crowded mitochondria were in the apical region. Other cell organelles in these tall cells were similar to those contained in the short cylindrical cells.

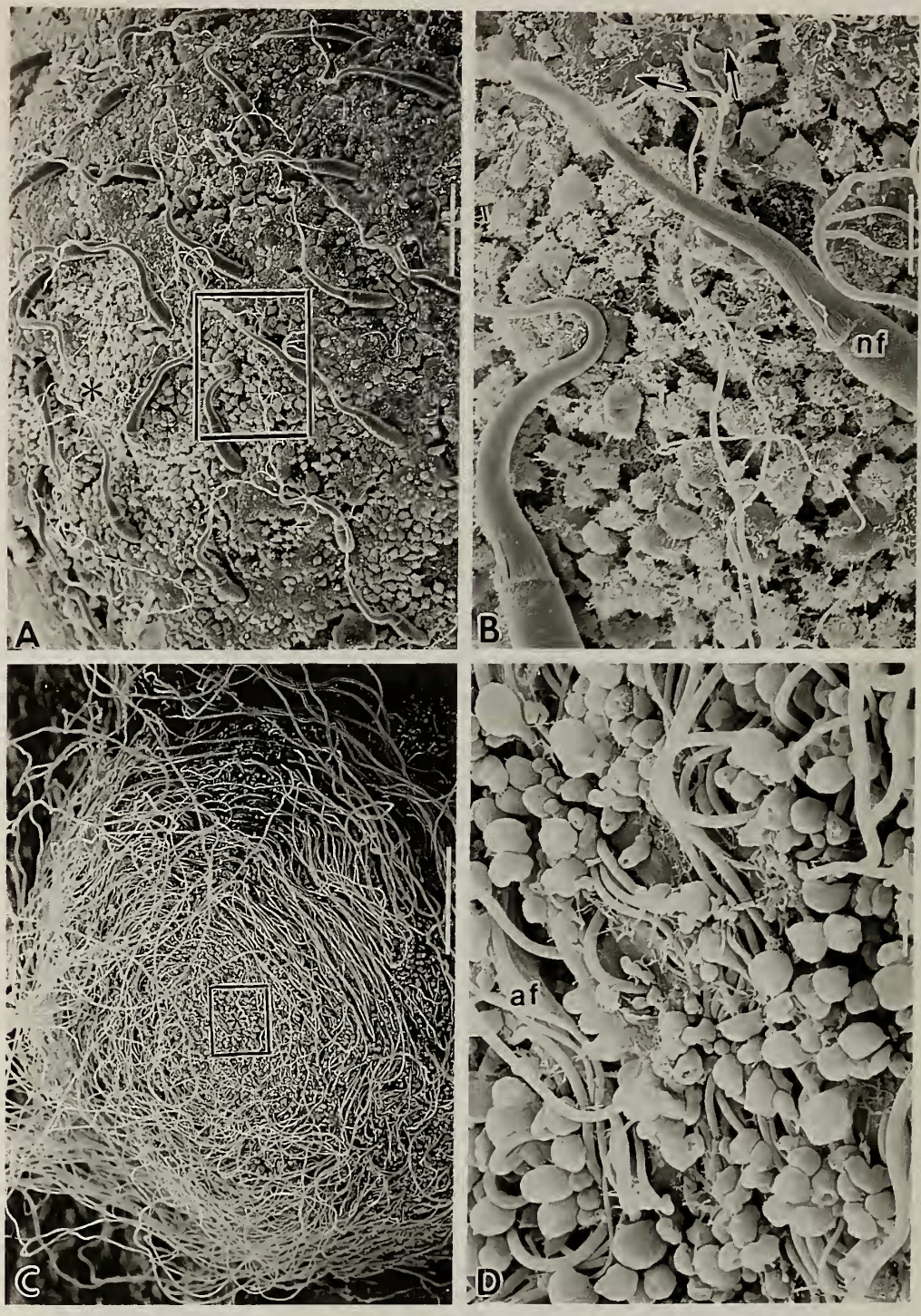


Fig. 3. Scanning electron micrographs of the surface of the granulosa cell layer in the animal and vegetal hemispheres. A, B: In the animal pole region (asterisk)(A; bar 50 $\mu$m), granulosa cells interact with each other by thread-like microvilli (B; bar 10 $\mu$m). nf, Non-attaching filaments; arrows, flat granulosa cells. C, D: In the vegetal pole region (asterisk) (C; bar 100 $\mu$m), which is encircled by the long distal portions of attaching filaments (af), the breb-like tips of the granulosa cells exhibit smooth surfaces (D; bar 10 $\mu$m) except for a few special cells with long microvilli.

The animal pole of the oocyte could be easily recognized by its position opposite the location of the attaching filaments on the chorion and by the bending direction of the non-attaching filaments (Fig. 3A). Granulosa cells at the animal pole region had many microvilli on the cell surface (Figs. 1 and 3B). The apical surface of these irregular-shaped granulosa cells was flat and smooth, as previously reported for the intact follicle [6, 7]. These flat granulosa cells were adherent to each other. The surface of the granulosa cells in the VPA is shown in Figs. 3C and D. In the VPA, tall granulosa cells

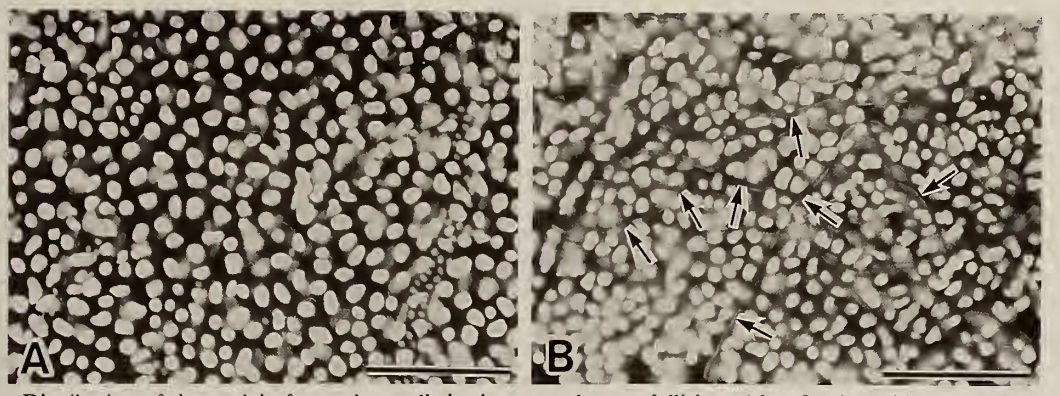


FIG. 4. Distribution of the nuclei of granulosa cells in the preovulataory follicle. After fixation with glutaraldehyde, the follicle was stained with 10 $\mu$g/ml Hoechst for 30 min (bar 100 $\mu$m). A: The animal hemisphere showing the nuclei of granulosa cells. B: The vegetal pole region showing the nuclei of granulosa cells and attaching filaments (arrows). Bars, 100 $\mu$m.

TABLE 1. Difference in steroid production by the animal and the vegetal hemispheres of medaka follicles

| Group | Estradiol-17$\beta$ (pg/ml) | | 17$\alpha$,20$\beta$-Dihydroxyprogesterone (pg/ml) | |
|---|---|---|---|---|
| | AH | VH | AH | VH |
| PMSG (100IU/ml) | 666±57 | 745±70 | 405±54 | 615±29* |
| Progesterone (100ng/ml) | 723±52 | 868±112 | 667±32 | 720±110 |
| Control (no hormone) | 200±28 | 300±49 | 6±4 | 31±29 |

* Significant difference ($P<0.05$) between the animal (AH) and vegetal (VH) hemispheres. In each group, values represent mean±S.E. of three incubations.

were compactly distributed, although it was difficult to precisely determine their size and shape by SEM.

The apical portions of the tall granulosa cells were lobular in shape (Figs. 2 and 3D). Many proximal portions of attaching filaments were observed in the VPA. The nuclei of the granulosa cells when observed by Hoechst staining appeared to avoid the areas of non-attaching filaments on the chorion (Fig. 4), because the nuclei existed out of focus. Calculated by the number of nuclei, the mean number of granulosa cells was about $1.5\times10^4$ in the animal hemisphere. This coincided with the value determined by calculation from the cell size (assuming the diameter as ca. 8 $\mu$m). The mean number of cells in the vegetal hemisphere including the VPA with its tall cells (mean diameter ca. 5 $\mu$m) was $1.7\times10^4$ as determined by the calculation from the cell size.

*2 Production of steroids by granulosa cells*

$E_2$ and 17$\alpha$,20$\beta$-diOHp produced by cultured granulosa cells were measured and the values for the animal and vegetal hemispheres were compared. The production of $E_2$ was stimulated by the presence of 100 IU/ml PMSG and 100 ng/ml progesterone (Table 1). No significant difference ($P>0.05$) in $E_2$ production was measured between the animal and the vegetal hemispheres. The production of 17$\alpha$,20$\beta$-diOHp by granulosa cells was also stimulated by the presence of PMSG or progesterone. The concentrations (more than 0.4 pg/ml; [9]) of 17$\alpha$,20$\beta$-diOHp were sufficient to induce oocyte maturation, and significantly greater in the vegetal hemisphere than in the animal hemisphere in the presence of PMSG.

## DISCUSSION

The granulosa cells are in contact with the oocyte via cytoplasmic processes that pass through the pore canals in the chorion in the medaka [2] and the pipefish [1]. The present observations revealed morphological differences in granulosa cells localized at special regions (VPA) surrounding the oocyte. These differences seem to reflect the different interactions of the granulosa cells with the oocyte, the polarity of which is established during oogenesis. In the early stage of oogenesis, the VPA is determined by the position of the Balbiani body in the ooplasm, and the granulosa cells aligned compactly on the VPA (Iwamatsu, unpublished data). The attaching filaments differentiate and elongate on a restricted region of the chorion in the VPA, and then spirally wind around the VPA probably due to rotation of the oocyte and granulosa cells [6].

We recently found that growing oocytes with granulosa cells may rotate within the basement membrane [5]. The granulosa cells in the animal pole region possess many microvilli on their apical surfaces, but those in the remaining area do not. The difference in the distribution of the

granulosa cells with microvilli may be related to a difference in the interactions with the basement membrane, or movement of granulosa cells beneath the basement membrane. Whether the morphological differences in the granulosa cells depend on regional differences in the chorion regions with which they are in contact is not clear from the present study, but it was suggested that the patterns of spiral structures on the chorion are due to the movement of follicular cells during oogenesis [6].

The steroidogenic response to exogenous hormones of granulosa cells from the animal pole hemisphere differed from that of cells from the vegetal hemisphere. The production of 17$\alpha$,20$\beta$-diOHp by granulosa cells that were stimulated by gonadotropin was greater in the vegetal hemisphere than in the animal hemisphere. A similar tendency was also observed in the steroid production by progesterone-stimulated follicles, although it was not significant statistically. The difference may be due to the difference in cell number, since there are more cells in the vegetal hemisphere than in the animal hemisphere. On the other hand, another cause of the difference may relate to the high ability of the tall granulosa cells with crowded mitochondria to produce steroids in the vegetal pole area. The cells that produce steroids are generally filled with mitochondria with well-developed tubular cristae and tubular or dilated ER (see [12]). However, no difference in $E_2$ production was recognized between the two hemispheres, in spite of the difference in cell number. This may indicate that the $E_2$ production by each granulosa cell in the vegetal hemisphere is not different from that in the animal hemisphere during the maturation period. Therefore, the granulosa cells with gonadotropin receptors seem to differ physiologically or distributively along the animal-vegetal axis of the oocyte.

## ACKNOWLEDGMENTS

The authors thank Dr. Cherrie A. Brown, California Regional Primate Research Center, University of California, Davis, for critical reading of the manuscript.

## REFERENCES

1 Begovac PC, Wallace RA (1989) Major vitelline envelope proteins in pipefish oocytes originate within the follicle and are associated with the Z3 layer. J Exp Zool 251: 56–73

2 Hirose K (1972) The ultrastructure of the ovarian follicle of medaka, *Oryzias latipes*. Z Zellforsch 123: 316–329

3 Iwamatsu T (1975) Medaka as a teaching material. II. Maturation and fertilization of oocytes. Bull. Aichi Univ. Educ., 24 (Nat. Sci.): 113–144 (in Japanese)

4 Iwamatsu T (1980) Studies on oocyte maturation of the medaka, *Oryzias latipes*. VIII. Role of follicle cells in gonadotropin- and steroid-induced maturation of oocytes *in vitro*. J Exp Zool 206: 355–364

5 Iwamatsu T (1992) Morphology of filaments on the chorion of oocytes and eggs in the medaka. Zool Sci 9: 589–599

6 Iwamatsu T, Nakashima S, Onitake K (1993) Spiral patterns in the micropylar wall and filaments on the chorion in eggs of the medaka, *Oryzias latipes*. J Exp Zool 267: 225–232

7 Iwamatsu T, Ohta T (1981) On a relationship between oocyte and follicle cells around the time of ovulation in the medaka, *Oryzias latipes*. Annot Zool Japon 54: 17–29

8 Iwamatsu T, Ohta T, Oshima E, Sakai N (1988) Oogenesis in the medaka *Oryzias latipes*. —Stages of oocyte development. Zool Sci 5: 353–373

9 Iwamatsu T, Takahashi SY, Sakai N, Asai K (1987) Inductive and inhibitory actions of a low molecular weight serum factor on *in vitro* maturation of oocytes of the medaka. Biomed Res 8: 313–322

10 Iwamatsu T, Takahashi SY, Sakai N, Onitake K (1987) Induction and inhibition of *in vitro* oocyte maturation and production of steroids in fish follicles by forskolin. J Exp Zool 241: 101–111

11 Masui Y, Clarke HJ (1979) Oocyte maturation. Intern Rev Cytol 57: 185–282

12 Nagahama Y (1983) Functional morphology of teleost gonads. In "Fish Physiology", vol. IXA (WS Hoar, AJ Randall and E M Donaldson, eds), pp. 223–275, Academic Press, New York

13 Nagahama Y, Adachi S (1985) Identification of maturation-inducing steroid in a teleost, the amago salmon (*Oncorhynchus rhodurus*). Develop Biol 109: 428–435

14 Riehl R, Appelbaum S (1991) A unique adhesion apparatus on the eggs of the catfish *Clarias gariepinus* (Teleostei, Clariidae). Jap J Ichthyol 38: 191–197

15 Sakai N, Iwamatsu T, Yamauchi K, Suzuki N, Nagahama Y (1988) Influence of follicular development on steroid production of the medaka (*Oryzias latipes*) ovarian follicle in response to exogenous substances. Gen Comp Endocrinol 71: 516–523

16 Schuetz AW (1967) Action of hormoness on germinal vesicle breakdown in frog (*Rana pipiens*) oocytes. J Exp Zool 166: 347–354